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Functional Integration of ES Cell-Derived Neurons *In Vivo*

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Abstract

Pluripotency and the potential for continuous self-renewal make embryonic stem (ES) cells an attractive donor source for neuronal cell replacement. Despite recent encouraging results in this field, little is known about the functional integration of transplanted ES cell-derived neurons on the single cell level. To address this issue, ES cell-derived neural precursors exhibiting neuron-specific EGFP expression were introduced into the developing brain. Donor cells implanted into the cerebral ventricles of embryonic rats migrated as single cells into a variety of brain regions, where they acquired complex morphologies and adopted excitatory and inhibitory neurotransmitter phenotypes. Synaptic integration was suggested by the expression of PSD-95 on donor cell dendrites, which in turn were approached by multiple synaptophysin-positive host axon terminals. Ultrastructural and electrophysiological data confirmed the formation of synapses between host and donor cells. Ten to 21 days after transplantation, all EGFP-positive donor cells examined displayed active membrane properties and received glutamatergic and GABAergic synaptic input from host neurons. These data demonstrate, at the single cell level, that grafted ES cell-derived neurons undergo morphological and functional integration into the host brain circuitry. Antibodies to region-specific transcription factors were employed to explore whether functional donor cell integration depends on the acquisition of a regional phenotype. Our data show that only a fraction of the incorporated neurons express homeobox genes typical of their engraftment site. Thus, an incorrect regional 'code' does not preclude morphological and synaptic integration of ES cell-derived neurons.

Introduction

Transplantation of neural precursors has developed into a key strategy for cell replacement in the central nervous system (CNS) (Rossi and Cattaneo, 2002). A major limitation of this approach is the availability of donor tissue. Studies involving clinical neurotransplantation have, so far, mostly relied on fetal brain tissue (Bjorklund and Lindvall, 2000; Freed et al., 2001; Lindvall, 2001). Approaches to bypass the shortage in donor tissue include expansion of neural stem cells in vitro by mitogen treatment (Reynolds and Weiss, 1992; Gage et al., 1995; Johe et al., 1996; Studer et al., 1998; Ostenfeld et al., 2000), the ex vivo introduction of growth-stimulating oncogenes (Shihabuddin et al., 1995; Snyder et al., 1995; Lundberg et al., 1997; Barresi et al., 2003), xenotransplantation (Fink et al., 2000), enhancement of endogenous adult neurogenesis (Kuhn et al., 1997; Magavi et al., 2000; Nakatomi et al., 2002) and attempts to recruit non-neural adult stem cells from other tissues (Brazelton et al., 2000; Mezey et al., 2000; Jiang et al., 2002; Mezey et al., 2003).

Recent advances in ES cell technology have opened an alternative, fascinating perspective to generate neural donor cells in unlimited quantities. Protocols have been established for the efficient generation of pan-neural, glial and neuronal-restricted precursors from ES cells (Okabe et al., 1996; Li et al., 1998; Brüstle et al., 1999; Mujtaba et al., 1999). Upon transplantation, ES cell-derived neural precursors incorporate into the CNS and differentiate into neurons and glia (Brüstle et al., 1997; McDonald et al., 1999; Liu et al., 2000; Reubinoff et al., 2001; Zhang et al., 2001).

Despite the broad experimental application of neuronal transplantation, few studies have addressed the functional integration of single neurons in the host CNS. The availability of transfectable fluorescent labels such as the enhanced green fluorescent protein (EGFP), has largely facilitated electrophysiological recordings from living donor cells. The results of recent

studies indicate that grafted fetal neural precursors and immortalized cell lines develop functional properties of postmitotic neurons (Auerbach et al., 2000; Englund et al., 2002). Functional studies on ES cell-derived neurons have, so far, focused primarily on cell culture experiments. In vitro, ES cell-derived neurons exhibit voltage-gated inward and outward currents, express functional neurotransmitter receptors and form synaptic contacts (Bain et al., 1995; Strübing et al., 1995; Finley et al., 1996; Strübing et al., 1997). A recent publication suggests that dopaminergic progenitors generated from ES cells in vitro and grafted into the striatum of 6-OHDA-lesioned rats give rise to localized clusters of dopaminergic neurons which display electrophysiological properties similar to endogenous cells (Kim et al., 2002).

In this study, we demonstrate, on the single cell level, that ES cell-derived neurons have the potential to functionally integrate into a large variety of brain regions. Yet, only a fraction of the incorporating donor neurons adopt a regionally appropriate transcription factor expression, indicating that functional integration overrides positional identity.

Material and Methods

ES cell differentiation. Tau::EGFP knock-in ES cells were generated by targeting the cDNA for EGFP in frame into exon 1 of the *Tau* gene, resulting in a fusion protein which consists of the first 31 amino acids of Tau and EGFP (Tucker et al., 2001). Due to the absence of microtubule-binding domains at the carboxyl terminus, the fluorescence signal is distributed throughout the cytoplasm. In vitro differentiation of ES cells was performed as described (Okabe et al., 1996). Briefly, ES cells were expanded on γ -irradiated mouse embryonic fibroblasts in DMEM (Life Technologies/ Invitrogen, Karlsruhe, Germany) supplemented with 20% fetal bovine serum (FBS; Biochrom AG seromed, Berlin, Germany), 1x MEM-nonessential amino acids (Life Technologies), 8 mg/l adenosine, 8.5 mg/l guanosine, 7.3

mg/l cytidine, 7.3 mg/l uridine, 2.4 mg/l thymidine, 0.1 mM 2-mercaptoethanol, 26 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (all Sigma, Taufkirchen, Germany) and 10^3 U/ml leukemia inhibitory factor (Chemicon, Hofheim, Germany). After passaging onto gelatin-coated dishes (0.1% gelatin; Sigma), ES cells were trypsinized and transferred to bacterial dishes allowing embryoid body (EB) formation. EBs were propagated for 4 days in the absence of LIF and subsequently plated onto tissue culture dishes. One day after plating, the medium was replaced by ITSFn, i.e. DMEM/F12 (Life Technologies) supplemented with 5 μ g/ml insulin, 50 μ g/ml human APO-transferrin (both Intergene, Purchase, NY, U.S.A.), 30 nM sodium selenite (Sigma), 2.5 μ g/ml fibronectin (Life Technologies) and penicillin/streptomycin (Life Technologies). After 5-7 days, cells were trypsinized, triturated to a single cell suspension and resuspended in Hank's buffered salt solution (HBSS) for transplantation.

Cell transplantation, immunohistochemical analysis and quantification. Surgery was performed as described (Brüstle et al., 1996). In brief, E16.5 timed pregnant Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were anesthetized with ketamine-HCl and xylazine (80 and 10 mg/kg bodyweight i.p., respectively). The uterine horns were exposed and the telencephalic vesicles of the embryos identified under transillumination. Using a glass capillary, $2-4 \times 10^5$ cells in a volume of 4-8 μ l HBSS were injected into one telencephalic vesicle. Injected embryos were placed back into the abdominal cavity for spontaneous delivery. One or 14 days after birth, animals were perfused transcardially with 4% paraformaldehyde. The brains were removed and cut on a vibratome into 50 μ m coronal sections or cryoprotected in 15% saccharose, frozen in isopentan and sectioned into 50 μ m slices. In some animals, obstruction of the ventricular system resulted in severe hydrocephalus. These animals were excluded from further analysis. Mouse monoclonal antibodies were used to detect β -III tubulin (1:500; Babco/ Covance, Richmond, California, U.S.A.), NeuN (1:200; Chemicon), GABA_A-receptors (β -chain, clone BD17; 1:50 Chemicon),

synaptophysin (1:40; DAKO, Hamburg, Germany), Pax6 (1:10; Developmental Studies Hybridoma Bank, Iowa City, Iowa, U.S.A.), and calbindin (1:5.000; SWant, Bellinzona, Switzerland). Primary rabbit antibodies were directed against GAD-65/67 (1:2.000; Chemicon), tyrosine hydroxylase (1:200; Affinity Research Products, Mamhead, U.K.), serotonin (1:2.000; Biogenesis, Poole, U.K.), the AMPA receptor subunit GluR1 (1:300; Sigma), the NMDA receptor subunit NR1 (1:1.000; Chemicon), and PSD-95 (a generous gift from Dr. M. Watanabe, Hokkaido University, Sapporo, Japan (Fukaya and Watanabe, 2000)). Dlx proteins were detected by using an antibody raised against the *Drosophila* Dll protein that binds to all vertebrate Dlx homologs (a generous gift from Dr. G. Panganiban, University of Wisconsin, Madison, Wisconsin (Panganiban et al., 1995)). A polyclonal goat antibody was used to detect the excitatory amino-acid carrier 1 (EAAC1), a neuronal glutamate transporter protein (1:8000; Chemicon). Primary antibodies were applied overnight in 1% normal goat serum in PBS. Antigens were visualized using appropriate rhodamine- or Cy-3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A.). Sections were preserved in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and observed on Zeiss Axioskop 2, Zeiss LSM510 and Leica TCS laser scan microscopes. The number of incorporated cells was determined in a minimum of three 50 μ m thick sections per animal and brain region. Incorporated neurons were defined as EGFP-positive cells located at least 50 μ m off the ventricular wall or a cluster of transplanted cells.

Electron microscopy. For preembedding immunohistochemistry, 50 μ m vibratome sections were incubated with a polyclonal antibody to EGFP (1:4.000; Abcam Ltd., Cambridge, U.K.), followed by a peroxidase-labeled secondary antibody (Jackson ImmunoResearch Laboratories Inc.) and staining with diaminobenzidine (DAB) precipitate (DAKO). Stained sections were postfixed overnight in 2.5% glutaraldehyde, photodocumented, dehydrated in graded ethanol series and embedded in Epon (Epoxy-Embedding Kit; Fluka Production

GmbH, Buchs, Switzerland) between two plastic slides overnight at 60°C. Regions of interest were microdissected into small fragments (approximately 1.5 x 3mm) and fixed onto an Epon block with a drop of liquid Epon following incubation at 60°C for >12 hours. Ultrathin sections were prepared on a Leica Ultracut Microtome, stained with uranyl acetate and lead citrate (10 min each) and observed on a Zeiss EM 900 electron microscope.

Slice preparation and electrophysiology. For electrophysiological recordings, recipient animals were decapitated under deep ether anaesthesia and 300 μ m horizontal sections were prepared using a vibratome (Leica VT1000S). Slices were transferred to the stage of an upright microscope (Zeiss Axioskop FS II) and perfused with ACSF containing (in mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 2.0 CaCl_2 , 1.0 MgCl_2 and 20 glucose (pH 7.3, NaOH) for current-clamp recordings as well as recordings of voltage-dependent membrane currents. For recordings of NMDA receptor-mediated excitatory postsynaptic currents (EPSCs), glycine (5 μ M) was added to the extracellular solution. In addition, MgCl_2 was omitted from the extracellular solution in some experiments. The blockers of synaptic transmission 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX, 50 μ M), bicuculline (10 μ M) and D(-)-2-amino-5-phosphonopentanoic acid (AP5, 50 μ M) were bath-applied.

EGFP-positive cells were readily identified using a fluorescence camera (Spot Jr., Diagnostic Instruments, Inc./Visitron Systems, Puchheim, Germany, see Fig. 3A₂), and these cells were subsequently visualized using infrared video microscopy and differential interference contrast optics in order to obtain patch-clamp recordings under visual control (Fig. 3A₁). For each recording, positive identification of the EGFP-positive donor cell was confirmed by diffusion of EGFP into the patch pipette (see Fig. 3A₃). Cells were recorded from the following incorporation sites: neocortex (n=15), hippocampus (n=12), inferior colliculus (n=11), thalamus (n=8), striatum (n=7), superior colliculus (n=6), amygdala (n=6), septum (n=4),

hypothalamus (n=4). For five cells, the incorporation site could not be attributed to a defined region.

Glass microelectrodes were pulled from borosilicate glass (2.0 mm diameter, wall thickness 420 μm) and had a resistance of 3.0-4.5 M Ω . For current-clamp recordings, pipettes contained (in mM): 20 KCl, 120 potassium gluconate, 10 ethyleneglykol-bis-(2-aminoethyl)-tetraacetic acid, 10 HEPES, 2 MgCl₂, 2 adenosine-5'-triphosphate disodium salt (ATP). For recordings of postsynaptic currents (PSCs), pipettes contained (in mM): 110 cesium methanesulfonate, 2 MgCl₂, 10 1,2-bis(2-amino-5-bromophenoxy)ethane-N,N,N',N'-tetraacetic acid, 2 ATP, 10 HEPES, 20 tetraethylammonium chloride, 5 QX314 (pH 7.4, NaOH). Liquid junction potentials were not compensated. All chemicals were purchased from Sigma.

Whole-cell voltage and current-clamp recordings were obtained at room temperature using a patch clamp amplifier (EPC9, HEKA Instruments, Lambrecht/Pfalz, Germany). After establishing the whole-cell configuration, the resting membrane potential and cell capacitance was measured. In all voltage-clamp recordings, the capacitance compensation circuitry of the patch-clamp amplifier was employed to reduce capacitive transients. Series resistance was on average 16.3 ± 1.8 , and was compensated by >70%. Traces were leak-subtracted on-line. PSCs were elicited by a 0.1 ms current pulse delivered via a monopolar glass stimulation electrode. For eliciting PSCs, stimulation electrodes were placed at a distance between 360 to 820 μm from the recorded cell. The vicinity of the stimulation electrode was carefully examined for the presence of EGFP-positive profiles, and only stimulation sites devoid of such profiles were used.

Data analysis. The time constants of PSC decay (τ) were determined by fitting a single exponential equation of the following form to the falling phase of the PSCs: $I(t) = A_0 + A_1 \cdot (1 -$

$\exp(-t/\tau)$) where $I(t)$ is the current amplitude at the time point t and A_0 is a constant offset. Fitting and determination of 10-90% rise times of PSCs was carried out using the program Igor (Wavemetric, USA). Spike threshold was defined as the membrane potential where the slope of the voltage trace increased abruptly during membrane charging induced by positive current pulses. Spike amplitude was measured as the voltage difference between the peak of the action potential and resting membrane potential (V_m). Spike width was calculated as spike duration at 50% of the spike amplitude.

Results

Grafted ES cell-derived neural precursors exhibit widespread migration and differentiation into multiple neuronal phenotypes

The neuron-specific EGFP fluorescence in progeny of tau::EGFP mutant ES cells provides a reliable system for the detection and live analysis of transplanted ES cell-derived neurons on the cellular level (Tucker et al., 2001; Wernig et al., 2002). Upon injection into the cerebral ventricles of day 16.5 rat embryos, neural precursors derived from heterozygote tau::EGFP knock-in ES cells formed intraventricular clusters and migrated as single cells to a large number of brain regions (Fig. 1, Table 1). Donor cell clusters within the ventricular system showed large variations with respect to size and content of EGFP-positive cells. Small, largely EGFP-positive clusters (Fig. 1A,B) contrasted with larger, tumorous formations with the histological appearance of teratomas. One to 3 weeks following transplantation, numerous donor-derived neurons had incorporated as single cells into thalamus, hypothalamus and tectum; neurons were also found engrafted in neocortex, olfactory bulb, amygdala, hippocampus, septum and striatum, although with less efficiency (Fig. 1A-D, Table 1). Sites exhibiting poor or lacking donor cell incorporation included cerebellum, ventral brain stem and medulla oblongata.

The strong EGFP fluorescence provided a crisp outline of the incorporated cells, clearly delineating their neuronal processes (Fig. 1E-L). EGFP-positive cells exhibited various neuronal morphologies, ranging from simple bipolar cells resembling young migratory neurons (Fig. 1E) to more complex phenotypes mimicking principal pyramidal neurons in the hippocampus (Fig. 1F) and multipolar neuronal cell types (Fig. 1G-L). Immunofluorescence analysis revealed that the engrafted donor cells had differentiated into various neurotransmitter subtypes (Fig. 2). One to 3 weeks after transplantation, $44.7 \pm 11.4\%$ of the incorporated EGFP-positive cells expressed the neuronal glutamate transporter protein

EAAC1 suggesting efficient differentiation into glutamatergic neurons ($n=1775$ cells analyzed in 10 recipients; Fig. 2A_{1,3}). A smaller population of $14.6\pm 10.1\%$ expressed glutamate decarboxylase (GAD-65/67), the rate-limiting enzyme for GABA synthesis indicating a GABAergic phenotype ($n=2237$ cells analyzed in 9 recipients; Fig. 2B_{1,3}). Some EGFP fluorescent cells were double-labeled with antibodies to tyrosine hydroxylase ($5.3\pm 8.2\%$; $n=2399$ cells analyzed in 9 brains), a marker for catecholaminergic neurons (Fig. 2C_{1,3}). Only occasionally, EGFP-positive donor cells were found double-labeled with antibodies to serotonin (Fig. 2D_{1,3}). Donor neurons incorporated into various host brain regions showed no striking differences in the distribution of excitatory and inhibitory neurotransmitter phenotypes (Table 2).

Incorporated ES cell-derived neurons exhibit passive and active membrane properties

To study the functional membrane properties of the engrafted neurons, acute slices were prepared from transplanted brains at postnatal days 10 to 21. Fluorescence microscopy permitted unequivocal identification of the incorporated EGFP-positive neurons. Patch-clamp recordings from donor cells ($n=78$) were obtained under visual control using infrared differential interference contrast optics. In each recording, diffusion of EGFP into the patch pipette after establishing the whole-cell configuration was used to confirm the donor cell identity (Fig. 3A_{1,3}). The resting membrane potential of ES cell-derived neurons ranged from -48 to -64 mV (average: $-55.2\text{mV}\pm 2.6\text{mV}$) with an input resistance of 205 ± 41 MOhm ($n=21$). Recordings in the current-clamp configuration allowed us to determine the active membrane characteristics of EGFP-positive cells. Prolonged (Fig. 3B₁) and brief (Fig. 3B₂) current injections demonstrated the capability to fire fast action potentials and action potential series (action potential amplitude ranging from 45 to 85 mV, average: $66.7\text{mV}\pm 4.9\text{mV}$, action potential half width ranging from 1.3 to 3.4 ms, average: 1.9 ± 0.2 , $n=19$). The transplanted

neurons also exhibited voltage-dependent membrane currents. Depolarizing voltage steps elicited both large outward currents with a sustained K^+ component (5.4 ± 1.5 nA) and fast inward Na^+ currents (4.4 ± 1.0 nA; Fig. 3C). These data demonstrate that ES cell-derived neurons exhibit typical neuronal membrane properties when transplanted into the rodent brain.

Synaptic integration of engrafted ES cell-derived neurons

To address the question whether the transplanted neurons receive synaptic contacts from host axons, we first examined the expression of the postsynaptic density protein (PSD-95). Confocal immunofluorescence analysis revealed a highly localized, punctate pattern within the grey matter as expected (Fukaya and Watanabe, 2000). In EGFP-expressing cells, PSD-95-positive puncta were typically found in close proximity to the cell surface, demonstrating that this major component of the postsynaptic density is expressed and correctly localized to the cell membrane (Fig. 4A). Next, we visualized presynaptic terminals with an antibody to synaptophysin. Numerous synaptophysin-positive, EGFP-negative patches were found in close apposition to the somatic and dendritic membranes of transplanted cells, suggesting that host-derived presynaptic terminals contact incorporated ES cell-derived neurons (Fig. 4B). Ultrastructural analysis was used to confirm synapse formation between host and donor neurons on the morphological level. Following preembedding immunohistochemistry with an antibody to EGFP, transplanted neurons were clearly detectable in the electron microscope. Careful examination of the donor cell membranes revealed postsynaptic densities in juxtaposition to EGFP-negative host-derived terminals containing synaptic vesicles (Fig. 4C).

We then tested whether incorporated EGFP-positive cells express ionotropic glutamate- and GABA-receptors, using antibodies to the GluR1 AMPA receptor subunit (Fig. 4D_{1,2}), the

GABA_A receptor β chain (Fig. 4E_{1,2}), and the NR1 subunit of the NMDA receptor (Fig. 4F_{1,2}). All subunits were clearly present in the majority of incorporated EGFP-positive cells, indicating that ES cell-derived neurons express both glutamatergic and GABAergic neurotransmitter receptors.

Finally, we sought to confirm functional synaptic integration of ES cell-derived neurons into the host brain. To this end, we elicited postsynaptic currents by stimulation with a monopolar stimulation electrode which was placed 360 to 820 μ m from the recorded cell. The vicinity of the stimulation electrode was carefully examined for the presence of EGFP-positive axonal or dendritic profiles to avoid stimulation of donor-derived neurons. When GABA_A and NMDA receptors were blocked by bicuculline (10 μ M) and AP5 (50 μ M), respectively, fast EPSCs were readily elicited. These EPSCs were completely abolished following additional application of 50 μ M CNQX, indicating that they were mediated by AMPA receptors (Fig. 5A₁, compare left and right panel, n=15). To estimate the reversal potential, the cell membrane was clamped at potentials ranging from -80 to +60 mV (Fig. 5A₂, n=4). The reversal potential was close to 0 mV (Fig. 5A₃). GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) were isolated pharmacologically by applying CNQX and AP5 (both 50 μ M, Fig. 5B₁, left panel). The resulting IPSCs were completely blocked following additional application of 10 μ M bicuculline (Fig. 5B₁, right panel, n=11). The reversal potential of GABAergic IPSCs was around -40 mV (Fig. 5B₂, B₃, n=5). Despite the abundant expression of NR1 within donor neurons, we were unable to detect EPSCs mediated by NMDA receptors (n=15 cells). Taken together, these morphological and electrophysiological data demonstrate that engrafted ES cell-derived neurons undergo functional synaptic integration into the host brain and receive both AMPA and GABA receptor-mediated input.

Ectopic expression of region-specific markers in transplanted cells

We next wondered whether and to what extent synaptic integration depends on region-specific differentiation of the donor neurons. Within neocortex and the CA1 pyramidal cell layer of the hippocampus, we detected EGFP-positive cells with pyramidal morphologies, appropriate dendritic orientation and expression of the neuronal glutamate transporter protein EAAC1, indicating elaboration of local excitatory phenotypes (Fig. 1F, 6A). Other donor neurons incorporated into the cortex displayed multipolar or tangential dendrites and expressed calbindin, i.e. features characteristic of cortical interneurons (Fig. 1G-H, 6B). While observations such as these might suggest region-specific differentiation, the large variability in donor and host cell phenotypes does not permit any conclusive statements. In fact, we also observed donor cell morphologies without obvious similarities to adjacent host neurons.

To more comprehensively characterize the positional identities of the transplanted neurons, we evaluated the expression of the homeotic genes *Dlx* and *Pax6*. During early development, *Dlx* genes are exclusively expressed in the ventral tel- and diencephalon including the ganglionic eminences, ventral thalamus as well as central and lateral areas of the hypothalamus. Their expression is maintained until at least early adulthood (Price et al., 1991; Liu et al., 1997; own observations). *Pax6* transcripts are detectable in the ventral part of the caudal neural tube, in di-, met- and myelencephalon, and in the lateral and dorsal parts of the telencephalon. *Pax6* is absent in the telencephalic ganglionic eminences and in the mesencephalic tectum (Walther and Gruss, 1991). Except the metencephalic expression domain, which is first observed at day 15.5 *p.c.*, this spatial expression pattern is maintained during brain development from E8.0 to E18.5. After midgestation, overall *Pax6* transcription gradually declines and becomes confined to discreet cerebral nuclei postnatally (Walther and Gruss, 1991; Stoykova and Gruss, 1994).

We used immunofluorescence analysis to study the expression of *Dlx* and *Pax6* in ES cell-derived neurons engrafted into cortex, striatum, hypothalamus and tectum. For detection of *Dlx* we used an antibody to the drosophila *Dll* protein, which binds to all vertebrate *Dlx*-homologs (Panganiban et al., 1995). Remarkably, only a small fraction of the incorporated cells was found to exhibit a regionally appropriate *Dlx* expression pattern (Fig. 6C,D; Table 2). In striatum, a region within the expression domain of *Dlx*, only 11 out of 163 cells showed immunoreactivity for *Dll* (n=2 animals; Fig. 6C). Conversely, donor-derived neurons incorporated in areas devoid of *Dlx* frequently exhibited ectopic expression of this marker. In tectum, a region lacking *Dlx* expression, 37% of EGFP-fluorescent cells were immunoreactive for *Dll* (n=7 animals; Fig. 6D).

Ectopic expression was also detected for *Pax6*. Five and 11% of the donor-derived neurons in the *Pax6*-negative territories striatum and tectum showed nuclear immunoreactivity, respectively (n=2 and n=7 animals). In neocortex and hippocampus, only 2% of the donor neurons expressed *Pax6* (based on 321 cells analyzed in 4 animals). Similarly, in diencephalon, only 23 out of 884 cells (n=4 animals) were found to be *Pax6*-positive. The latter findings have to be interpreted with care as *Pax6* is known to be down-regulated in most postmitotic neurons.

Discussion

Synaptic integration of transplanted ES cell-derived neurons

The results of this study demonstrate, on a single cell level, that engrafted ES cell-derived neurons are capable to synaptically integrate into the host brain circuitry. Upon transplantation into the cerebral ventricles of embryonic rats, ES cell-derived neural precursor cells efficiently migrate into multiple brain regions where they differentiate into cells exhibiting morphological and electrophysiological properties typical of neurons. Furthermore, the incorporated donor neurons receive both excitatory and inhibitory synaptic input from host axons.

Functional integration is regarded as a key prerequisite for neuronal replacement strategies. Previous studies have provided ultrastructural and first electrophysiological evidence that neurons derived from grafted fetal brain tissue can form synaptic contacts with host neurons (Freund et al., 1985; Segal et al., 1985; Bolam et al., 1987; Clarke et al., 1988; Wictorin et al., 1990). More recent investigations showed on the cellular level that fetal and immortalized neural stem cells develop into functional neurons and receive synaptic input from endogenous cells when transplanted to the embryonic or neonatal rat brain (Auerbach et al., 2000; Englund et al., 2002).

During the last 5 years, ES cells have emerged as an attractive new donor source for cell replacement strategies (Thomson and Marshall, 1998; Svendsen and Smith, 1999). It is, however, not known whether neurons derived by in vitro differentiation of ES cells exhibit functional properties in vivo. Observations by Kim et al. (2002) indicate that ES cell-derived neurons are capable to project onto host neurons upon transplantation into an animal model of Parkinson's disease. While no direct connections between donor and host cells were detected in paired recordings, extracellular stimulation within the striatal grafts resulted in

EPSPs in endogenous stellate neurons. In addition, extracellular stimulation was found to elicit IPSPs in donor neurons, suggesting synaptic input onto the transplanted cells. The interpretations of these observations are complicated by the lack of an intravital label, precluding the identification of donor-derived processes and a clear distinction between endogenous and grafted cells at the time of electrophysiological assessment.

In the current study, this critical point is bypassed by using ES cells engineered to express EGFP in a neuron-specific manner, permitting the prospective identification of engrafted donor-derived neurons and their processes (Tucker et al., 2001; Wernig et al., 2002). To be able to study individual donor neurons in various host brain regions, we abandoned classic intraparenchymal injection and merely implanted the cells into the ventricle of embryonic recipients. Typically, cells grafted in this manner enter the ventricular zone, from where they migrate into a large variety of brain regions (Campbell et al., 1995; Fishell, 1995; Brüstle et al., 1995). The highly dispersed incorporation pattern permits the analysis of donor neurons engrafted as single cells into areas devoid of other donor cells. Due to the early time point of implantation, this method further permits xenotransplantation without the necessity of immunosuppression (Brüstle et al., 1995; Campbell et al., 1995; Brüstle et al., 1998).

Following intraventricular deposition, ES cell-derived neurons, indeed, incorporated as single cells into the host brain parenchyma. They exhibited a broad spectrum of neuronal morphologies ranging from bipolar to complex multipolar phenotypes. Most cells differentiated into glutamatergic and GABAergic, some into catecholaminergic and only a small number into serotonergic subtypes. Electrophysiological assessment demonstrated that all incorporated neurons analyzed displayed active membrane properties. Donor neurons expressed proteins essential for synaptic transmission, including the postsynaptic density protein (PSD-95) and the major subunits of ionotropic glutamate and GABA receptors (NR1, GluR1 and GABA_A receptor β -chain, respectively). Synaptophysin-reactive

dots were found surrounding somata and dendrites of transplanted neurons, suggesting the presence of presynaptic terminals adjacent to their membranes. Synapse formation between host and donor neurons was confirmed by ultrastructural and functional studies. Extracellular stimulation of host neurons and patch-clamp recordings from incorporated donor neurons yielded both AMPA and GABA_A receptor-mediated postsynaptic currents in the engrafted cells. In contrast, we found no evidence for EPSCs mediated by NMDA receptors. Yet, many donor neurons were decorated with an antibody against the NR1 subunit of the NMDA receptor. The reasons for this striking discrepancy remain unclear, but a similar scarceness of NMDA receptor-mediated responses has been observed in cell culture experiments (Finley et al., 1996) and in previous studies on the functional integration of ES cell-derived neurons in cultured hippocampal slices (Benninger et al., 2003).

The transplanted cells not only gave rise to neurons incorporating into the host brain but also formed large intraventricular clusters containing, in addition to EGFP-positive cells, primitive neuroepithelial structures and areas of non-neural differentiation. These and earlier findings (Brüstle et al., 1997) indicate that ES cell-derived neural precursors generated according to the protocol of Okabe et al. (1996) still contain a fraction of undifferentiated cells capable of teratoma formation. Although further propagation in defined growth factor-containing media and lineage selection strategies can be employed to enrich neural cells and eliminate undifferentiated cells (Li et al., 1998; Brüstle et al., 1999; Mujtaba et al., 1999; Carpenter et al., 2003), we decided to employ immature, highly migratory cell populations at an early stage of differentiation for this study.

Region-specific differentiation is no prerequisite for functional integration

In the past, intraventricular transplantation into the developing rodent brain has been used to study the plasticity of fetal neural precursors. These studies have shown that neural progenitors derived from the E12-14 mouse brain can undergo widespread migration and regional differentiation (Campbell et al., 1995; Fishell, 1995; Brüstle et al., 1995). Studies involving ultrasound-guided intrauterine transplantation into specific brain regions suggest that the potential for region-specific differentiation depends primarily on the developmental stage of the transplanted cells. While E10.5 mid-hindbrain progenitors still have the capacity to induce *Nkx-2.1* expression when transplanted into the medial ganglionic eminence, E13.5 progenitors from the same region fail to induce region-specific genes upon heterotopic engraftment (Olsson et al., 1997).

Remarkably, an inappropriate regional transcription factor code appears not to preclude migration and incorporation in a heterotopic environment (Olsson et al., 1997; Na et al., 1998). The question arises whether and to what extent a heterotopic positional code impairs function, network integration and long-term survival of transplanted neurons. Telencephalic progenitors incorporated into the di- and mesencephalon fail to downregulate anterior markers and survive until adulthood (Na et al., 1998). In contrast, diencephalic precursors incorporated into cortex appear to die early postnatally (McCarthy et al., 2001). It is tempting to speculate that during development, heterotopic cells are barred from functional integration to protect local circuits from irritating activity.

Our embryonic transplantation paradigm allowed us to address these questions in detail. To our surprise, most incorporated cells did not adopt a region-specific transcription factor code. A large number of donor-derived neurons failed to induce *Dlx* in *Dlx*-positive territories or expressed this transcription factor in heterotopic locations. Similarly, the engrafted EGFP-

positive cells frequently showed Pax6 immunoreactivity in areas outside the Pax6 domains. The lacking acquisition of a local transcription factor code contrasted with the high efficiency of functional integration: All incorporated neurons analyzed displayed characteristic intrinsic membrane excitability, and — in all cases in which this property was studied — received synaptic input from host cells. Thus, faulty expression of positional genes does not appear to preclude functional neuronal maturation or synaptic integration.

Yet, a significant fraction of the transplanted neurons *did* express regionally appropriate transcription factors. Site-specific differentiation of engrafted neurons is also supported by the detection of regional phenotypes such as glutamatergic pyramidal neurons in cortex and hippocampus or calbindin-positive neurons with morphologies reminiscent of cortical interneurons. On the other hand, overall neurotransmitter expression of the engrafted neurons revealed no regional preferences.

The limited extent of regional differentiation might be due to several reasons. It is conceivable that only a subpopulation of the transplanted progenitors is susceptible to regionalizing host signals. This would imply that, despite their morphological and immunohistochemical homogeneity (Okabe et al., 1996), ES cell-derived neural precursors represent a rather heterogeneous population. As suggested by the studies of Olsson et al. (1997), regional plasticity might be largely restricted to very immature neural cells at early differentiation stages. It has been hypothesized that heterotopically grafted cells might have to undergo their last division in their new environment in order to respond appropriately to local cues (Brüstle et al., 1995). Additional studies on differentiating ES cell cultures will be required to define the optimal 'window' of susceptibility to extrinsic cues imposing a regional phenotype.

Our data demonstrate that engrafted ES cell-derived neural precursors have the capacity to develop into functional neurons, which integrate synaptically into the host brain circuitry. These findings represent an essential basis for the development of ES cell-based neuronal repair strategies. However, the observation that synaptic integration of ES cell-derived neurons does not depend on the acquisition of a region-specific transcription factor code indicates that functional integration is not strongly regulated by regional signals. This finding should be taken as a note of caution. It argues against a regional 'gating' mechanism, which protects local circuits from the integration of non-regionalized, potentially disruptive neurons and strongly emphasizes the need for regional pre-specification of the donor cells.

References

- Auerbach JM, Eiden MV, McKay RD (2000) Transplanted CNS stem cells form functional synapses in vivo. *Eur J Neurosci* 12:1696-1704.
- Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI (1995) Embryonic stem cells express neuronal properties in vitro. *Dev Biol* 168:342-357.
- Barresi V, Belluardo N, Sipione S, Mudo G, Cattaneo E, Condorelli DF (2003) Transplantation of prodrug-converting neural progenitor cells for brain tumor therapy. *Cancer Gene Ther* 10:396-402.
- Benninger F, Beck H, Wernig M, Tucker KL, Brüstle O, Scheffler B (2003) Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. *J Neurosci* 23:7075-7083.
- Bjorklund A, Lindvall O (2000) Cell replacement therapies for central nervous system disorders. *Nat Neurosci* 3:537-544.
- Bolam JP, Freund TF, Bjorklund A, Dunnett SB, Smith AD (1987) Synaptic input and local output of dopaminergic neurons in grafts that functionally reinnervate the host neostriatum. *Exp Brain Res* 68:131-146.
- Brazelton TR, Rossi FM, Keshet GI, Blau HM (2000) From Marrow to Brain: Expression of Neuronal Phenotypes in Adult Mice. *Science* 290:1775-1779.
- Brüstle O, Maskos U, McKay RD (1995) Host-guided migration allows targeted introduction of neurons into the embryonic brain. *Neuron* 15:1275-1285.
- Brüstle O, Cunningham M, Tabar V, McKay R (1996) Experimental transplantation in the embryonic, neonatal, and adult mammalian brain. In: *Current Protocols in Neuroscience* (Crawley J, Gerfen C, McKay R, Rogawski M, Sibley D, Skolnick P, eds), pp 13.10.11-28. New York: John Wiley.

- Brüstle O, Spiro AC, Karram K, Choudhary K, Okabe S, McKay RD (1997) In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci U S A* 94:14809-14814.
- Brüstle O, Choudhary K, Karram K, Huttner A, Murray K, Dubois-Dalcq M, McKay RD (1998) Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. *Nat Biotechnol* 16:1040-1044.
- Brüstle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD (1999) Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285:754-756.
- Campbell K, Olsson M, Bjorklund A (1995) Regional incorporation and site-specific differentiation of striatal precursors transplanted to the embryonic forebrain ventricle. *Neuron* 15:1259-1273.
- Carpenter MK, Rosler E, Rao MS (2003) Characterization and differentiation of human embryonic stem cells. *Cloning Stem Cells* 5:79-88.
- Clarke DJ, Dunnett SB, Isacson O, Sirinathsinghji DJ, Bjorklund A (1988) Striatal grafts in rats with unilateral neostriatal lesions--I. Ultrastructural evidence of afferent synaptic inputs from the host nigrostriatal pathway. *Neuroscience* 24:791-801.
- Englund U, Bjorklund A, Wictorin K, Lindvall O, Kokaia M (2002) Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. *Proc Natl Acad Sci U S A* 99:17089-17094.
- Fink JS, Schumacher JM, Elias SL, Palmer EP, Saint-Hilaire M, Shannon K, Penn R, Starr P, VanHorne C, Kott HS, Dempsey PK, Fischman AJ, Raineri R, Manhart C, Dinsmore J, Isacson O (2000) Porcine xenografts in Parkinson's disease and Huntington's disease patients: preliminary results. *Cell Transplant* 9:273-278.
- Finley MF, Kulkarni N, Huettner JE (1996) Synapse formation and establishment of neuronal polarity by P19 embryonic carcinoma cells and embryonic stem cells. *J Neurosci* 16:1056-1065.

- Fishell G (1995) Striatal precursors adopt cortical identities in response to local cues. *Development* 121:803-812.
- Freed CR, Greene PE, Breeze RE, Tsai WY, DuMouchel W, Kao R, Dillon S, Winfield H, Culver S, Trojanowski JQ, Eidelberg D, Fahn S (2001) Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med* 344:710-719.
- Freund TF, Bolam JP, Bjorklund A, Stenevi U, Dunnett SB, Powell JF, Smith AD (1985) Efferent synaptic connections of grafted dopaminergic neurons reinnervating the host neostriatum: a tyrosine hydroxylase immunocytochemical study. *J Neurosci* 5:603-616.
- Fukaya M, Watanabe M (2000) Improved immunohistochemical detection of postsynaptically located PSD-95/SAP90 protein family by protease section pretreatment: a study in the adult mouse brain. *J Comp Neurol* 426:572-586.
- Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci U S A* 92:11879-11883.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418:41-49.
- Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev* 10:3129-3140.
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50-56.

- Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci* 17:5820-5829.
- Li M, Pevny L, Lovell-Badge R, Smith A (1998) Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr Biol* 8:971-974.
- Lindvall O (2001) Parkinson disease. Stem cell transplantation. *Lancet* 358 Suppl:S48.
- Liu JK, Ghattas I, Liu S, Chen S, Rubenstein JL (1997) Dlx genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. *Dev Dyn* 210:498-512.
- Liu S, Qu Y, Stewart TJ, Howard MJ, Chakraborty S, Holekamp TF, McDonald JW (2000) Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc Natl Acad Sci U S A* 97:6126-6131.
- Lundberg C, Martinez-Serrano A, Cattaneo E, McKay RD, Bjorklund A (1997) Survival, integration, and differentiation of neural stem cell lines after transplantation to the adult rat striatum. *Exp Neurol* 145:342-360.
- Magavi SS, Leavitt BR, Macklis JD (2000) Induction of neurogenesis in the neocortex of adult mice. *Nature* 405:951-955.
- McCarthy M, Na E, Neyt C, Langston A, Fishell G (2001) Calcium-dependent adhesion is necessary for the maintenance of prosomeres. *Dev Biol* 233:80-94.
- McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW (1999) Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* 5:1410-1412.
- Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR (2000) Turning Blood into Brain: Cells Bearing Neuronal Antigens Generated in Vivo from Bone Marrow. *Science* 290:1779-1782.

- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B (2003) Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* 100:1364-1369.
- Mujtaba T, Piper DR, Kalyani A, Groves AK, Lucero MT, Rao MS (1999) Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev Biol* 214:113-127.
- Na E, McCarthy M, Neyt C, Lai E, Fishell G (1998) Telencephalic progenitors maintain anteroposterior identities cell autonomously. *Curr Biol* 8:987-990.
- Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110:429-441.
- Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD (1996) Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* 59:89-102.
- Olsson M, Campbell K, Turnbull DH (1997) Specification of mouse telencephalic and mid-hindbrain progenitors following heterotopic ultrasound-guided embryonic transplantation. *Neuron* 19:761-772.
- Ostenfeld T, Caldwell MA, Prowse KR, Linskens MH, Jauniaux E, Svendsen CN (2000) Human neural precursor cells express low levels of telomerase in vitro and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. *Exp Neurol* 164:215-226.
- Panganiban G, Sebring A, Nagy L, Carroll S (1995) The development of crustacean limbs and the evolution of arthropods. *Science* 270:1363-1366.
- Price M, Lemaistre M, Pischetola M, Di Lauro R, Duboule D (1991) A mouse gene related to *Distal-less* shows a restricted expression in the developing forebrain. *Nature* 351:748-751.

Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T (2001)

Neural progenitors from human embryonic stem cells. *Nat Biotechnol* 19:1134-1140.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of

the adult mammalian central nervous system. *Science* 255:1707-1710.

Rossi F, Cattaneo E (2002) Opinion: neural stem cell therapy for neurological diseases:

dreams and reality. *Nat Rev Neurosci* 3:401-409.

Segal M, Bjorklund A, Gage FH (1985) Transplanted septal neurons make viable cholinergic

synapses with a host hippocampus. *Brain Res* 336:302-307.

Shihabuddin LS, Hertz JA, Holets VR, Whittemore SR (1995) The adult CNS retains the

potential to direct region-specific differentiation of a transplanted neuronal precursor cell line. *J Neurosci* 15:6666-6678.

Snyder EY, Taylor RM, Wolfe JH (1995) Neural progenitor cell engraftment corrects

lysosomal storage throughout the MPS VII mouse brain. *Nature* 374:367-370.

Stoykova A, Gruss P (1994) Roles of Pax-genes in developing and adult brain as suggested

by expression patterns. *J Neurosci* 14:1395-1412.

Strübing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM (1995)

Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 53:275-287.

Strübing C, Rohwedel J, Ahnert-Hilger G, Wiedenmann B, Hescheler J, Wobus AM (1997)

Development of G protein-mediated Ca²⁺ channel regulation in mouse embryonic stem cell-derived neurons. *Eur J Neurosci* 9:824-832.

Studer L, Tabar V, McKay RD (1998) Transplantation of expanded mesencephalic

precursors leads to recovery in parkinsonian rats. *Nat Neurosci* 1:290-295.

Svendsen CN, Smith AG (1999) New prospects for human stem-cell therapy in the nervous

system. *Trends Neurosci* 22:357-364.

-
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.
- Tucker KL, Meyer M, Barde YA (2001) Neurotrophins are required for nerve growth during development. *Nat Neurosci* 4:29-37.
- Walther C, Gruss P (1991) Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* 113:1435-1449.
- Wernig M, Tucker KL, Gornik V, Schneiders A, Buschwald R, Wiestler OD, Barde YA, Brüstle O (2002) Tau EGFP embryonic stem cells: an efficient tool for neuronal lineage selection and transplantation. *J Neurosci Res* 69:918-924.
- Wictorin K, Clarke DJ, Bolam JP, Bjorklund A (1990) Fetal striatal neurons grafted into the ibotenate lesioned adult striatum: efferent projections and synaptic contacts in the host globus pallidus. *Neuroscience* 37:301-315.
- Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA (2001) In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 19:1129-1133.

TABLES AND FIGURES

Table 1: Incorporation of ES cell-derived neurons into the developing rat brain

animal	OB	NCX	HPC	AMG	SPT	STR	TH	HT	TCT	CB
P1	+	+		+			+	+	(+)	+
P1								(+)		
P1								+	+	
P1		(+)								
P1		(+)						+	+	
P1	(+)	+						+	+	
P1		(+)							+	
P1		(+)				(+)				
P1	+				+		+	+	++	
P14		+	+		+		+	+	++	(+)
P14						+			+	
P14								(+)	(+)	
P14			+	+		(+)			+	
P14		+				+	+	+	+	
P14							++	+	++	(+)
P14		+	+	+				(+)	(+)	
P14	+	+		+		+	(+)	(+)		
P14				+			+	+	(+)	

The distribution of the engrafted neurons showed pronounced inter- and intraindividual variations. (+): < 5 cells; +: 5-50 cells; ++: > 50 EGFP-positive cells per region and 50 μ m section. A minimum of 3 sections per region and animal were analyzed. P, postnatal day; OB, olfactory bulb; NCX, neocortex; HPC, hippocampus; AMG, amygdala; SPT, septum; STR, striatum; TH, thalamus; HT, hypothalamus; TCT, tectum; CB, cerebellum.

Table 2: Distribution of excitatory and inhibitory neurotransmitter phenotypes and Dlx expression in engrafted ES cell-derived neurons

animal	CTX	STR	DI	TCT
EAAC1	P1		8% (n=59)	59% (n=164)
	P1	56% (n=136)	38% (n=16)	45% (n=40)
	P1			55% (n=20)
	P1		21% (n=52)	33% (n=404)
	P14	84% (n=19)	42% (n=31)	n.d.
	P14	40% (n=82)	22% (n=156)	14% (n=42)
	P14	47% (n=34)	40% (n=217)	28% (n=89)
	P14	38% (n=50)	45% (n=56)	38% (n=52)
GAD-65/67	P1		10% (n=30)	14% (n=283)
	P1	56% (n=66)	20% (n=15)	0% (n=33)
	P1			15% (n=39)
	P1		17% (n=91)	12% (n=474)
	P14	3% (n=147)	38% (n=77)	n.d.
	P14	16% (n=178)	14% (n=70)	2% (n=138)
	P14	0% (n=44)	14% (n=286)	20% (n=50)
	P14	0% (n=53)	2% (n=46)	9% (n=33)
DLX	P1		6% (n=77)	7% (n=85)
	P1	0% (n=92)	13% (n=40)	19% (n=206)
	P1			12% (n=32)
	P1		10% (n=122)	36% (n=147)
	P14	7% (n=61)	n.d.	86% (n=103)
	P14	0% (n=118)	10% (n=79)	46% (n=452)
	P14	n.d.	1% (n=150)	21% (n=135)
	P14	0% (n=35)	4% (n=84)	0% (n=92)

The generation of glutamatergic and GABAergic neurons and the expression of Dlx proteins in engrafted ES cell-derived neurons showed high interindividual variations and was not pronounced in characteristic regions. CTX, cortex (including neocortex and hippocampus); DI, diencephalon; TCT, tectum; n.d., not determined. A minimum of 2 sections containing EGFP-positive cells were analyzed per region and animal. n represents the total number of EGFP-positive cells analyzed.

Figure 1:

Incorporation of ES cell-derived neurons into the developing rat brain. Engrafted donor cells identified by virtue of their EGFP fluorescence (green) or immunofluorescence with an antibody to EGFP (red) generate a variety of neuronal phenotypes. **A**, Twenty days following transplantation into the ventricle of E16.5 rats, the cells have formed intraventricular clusters and migrated as single cells into various host brain regions. **B-D**, higher power microphotographs of areas indicated in A, depicting incorporation into neocortex (B) and hypothalamus (D). Donor-derived cortical neurons were found to extend long axons into the corpus callosum (C). **E-L**, Confocal microscopy and digital reconstruction revealed that the transplanted cells adopt a variety of morphologies including simple bipolar cells resembling young migratory neurons (E, neocortex), complex phenotypes mimicking principal pyramidal neurons of the hippocampus (F, CA1 pyramidal cell layer) and multipolar cell types (G-H, neocortex; I, septum; J, thalamus; K-L, tectum). Scale bars: 1 mm (A); 200 μm (B); 100 μm (C-D); 50 μm (E-L).

Figure 2:

Integrated ES cell-derived neurons differentiate into various neurotransmitter subtypes. Confocal microscopy demonstrated that many EGFP-positive neurons were double labeled with antibodies to EAAC1, suggesting a glutamatergic phenotype (**A**, cortex) and GAD-65/67, the rate limiting enzyme for GABA synthesis (**B**). A smaller fraction of EGFP-positive cells expressed tyrosin hydroxylase (TH; **C**, thalamus) or serotonin (5 HT; **D**, thalamus). Scale bar: 20 μm .

Figure 3:

Intrinsic discharge properties and voltage-gated membrane currents observed in engrafted ES cell-derived neurons 20 days after intrauterine transplantation. **A**, Infrared differential interference contrast image (A_1) and fluorescent image (A_2) of an EGFP-positive donor neuron after formation of a gigaseal. In all recordings, diffusion of EGFP into the pipette served as confirmation of the donor cell identity (A_3). **B**, Current-clamp recordings during prolonged (B_1) and brief (B_2) current injections. Top traces represent voltage recordings, whereas bottom traces indicate current injections. To facilitate discrimination of voltage traces, part of the traces and their corresponding current injection steps are shown in grey. **C**, Voltage-dependent membrane currents. Depolarizing voltage-steps (bottom traces, potentials as indicated) elicited outward currents with a sustained K^+ component and fast inward Na^+ currents.

Figure 4:

Engrafted ES cell-derived neurons exhibit morphological and immunohistochemical evidence of synapse formation. **A**, PSD-95-immunoreactive puncta on the cell membrane of an EGFP-fluorescent cell (arrow heads). **B**, Synaptophysin-positive presynaptic terminals contacting soma and dendrites of an incorporated ES cell-derived neuron. **C**, Electron micrograph revealing synapse formation between an EGFP-negative terminal containing presynaptic vesicles (yellow arrow head) and the soma of an EGFP-immunoreactive ES cell-derived neuron (red dotted line marks border of the EGFP-immunoreactive donor cell). Insert: higher magnification micrograph from (C). **D-F**, Engrafted neurons express receptors for excitatory and inhibitory neurotransmitters including the $GABA_A$ receptor β -chain (D, tectum), the GluR1 subunit of the AMPA receptor (E, hypothalamus) and the NR1 subunit of the NMDA receptor (F, tectum). A, B, and D-F represent single $1.2\ \mu m$ confocal planes. Scale bars: $10\ \mu m$ (A, B, D-F); $0.4\ \mu m$ (C).

Figure 5:

Excitatory and inhibitory synaptic transmission from host to ES cell-derived neurons. **A**, AMPA receptor-mediated EPSCs. Extracellular stimulation of host neurons readily evoked fast EPSCs in the presence of bicuculline ($10\mu\text{M}$) and AP5 ($50\mu\text{M}$) (A_1 , leftmost traces). EPSCs were completely abolished after additional application of the AMPA receptor blocker CNQX ($50\mu\text{M}$) (A_1 , rightmost traces). To estimate the reversal potential, the cell membrane was clamped at different voltages (-80 to $+40\text{mV}$ as indicated) and AMPA receptor-mediated EPSCs were elicited by synaptic stimulation. The reversal potential was close to 0 mV (A_2 , A_3). **B**, GABA_A receptor-mediated synaptic input. Slow IPSCs were elicited following stimulation in the presence of $50\mu\text{M}$ CNQX and AP5 (B_1 , leftmost traces). These currents were completely abolished after additional application of $10\mu\text{M}$ bicuculline (B_1 , rightmost traces). Analogous to panel A, the cell membrane was clamped at different potentials as indicated and GABA_A receptor-mediated IPSCs were elicited by stimulation with a monopolar stimulation electrode. The reversal potential was approximately -40mV (B_2 , B_3).

Figure 6:

Evidence for site-specific and ectopic neuronal differentiation of ES cell-derived neural precursors following transplantation to the embryonic cerebral ventricles. **A**, An EGFP-fluorescent cell within the neocortex of a 14-day-old rat brain exhibiting a pyramidal morphology, appropriate orientation of the dendritic tree (A_1) and expression of the neuronal glutamate transporter protein EAAC1 (red, A_2). **B**, An ES cell-derived donor neuron incorporated into the cortex of an 1-day-old animal with tangentially orientated dendrites (B_1), expressing the interneuron marker calbindin (red, B_2). The red pointers in A and B direct to the pial surface. Some EGFP-positive neurons within the striatum exhibited site-specific

expression of Dlx (**C**_{1,2}). However, Dlx proteins were also present in ES cell-derived neurons incorporated into the tectum (arrowheads, arrow indicates a Dlx-negative cell), an area devoid of endogenous *Dlx* expression (**D**_{1,2}). **A**₁, **B-C** represent digital reconstructions following confocal microscopy, **A**₂ represents a single 1.2 μm confocal plane. Scale bars: 50 μm (**A-C**); 100 μm (**D**).

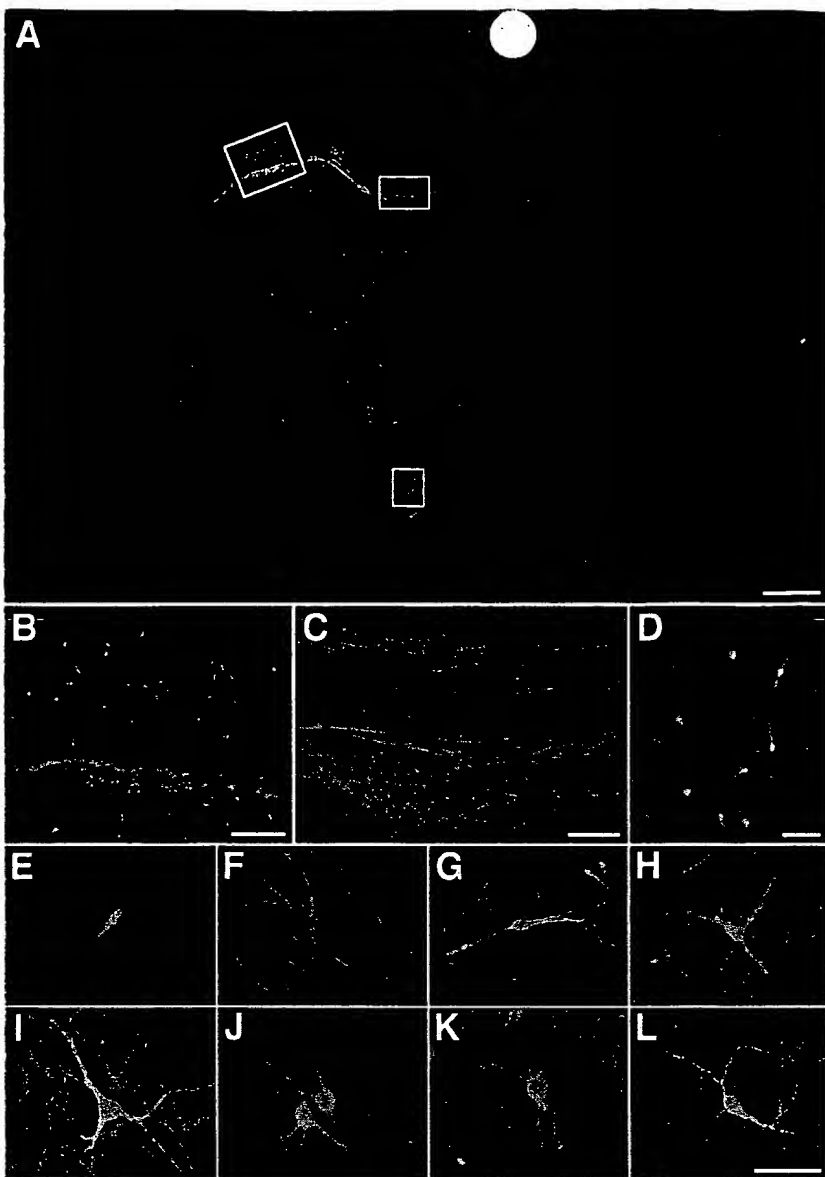


FIGURE 1

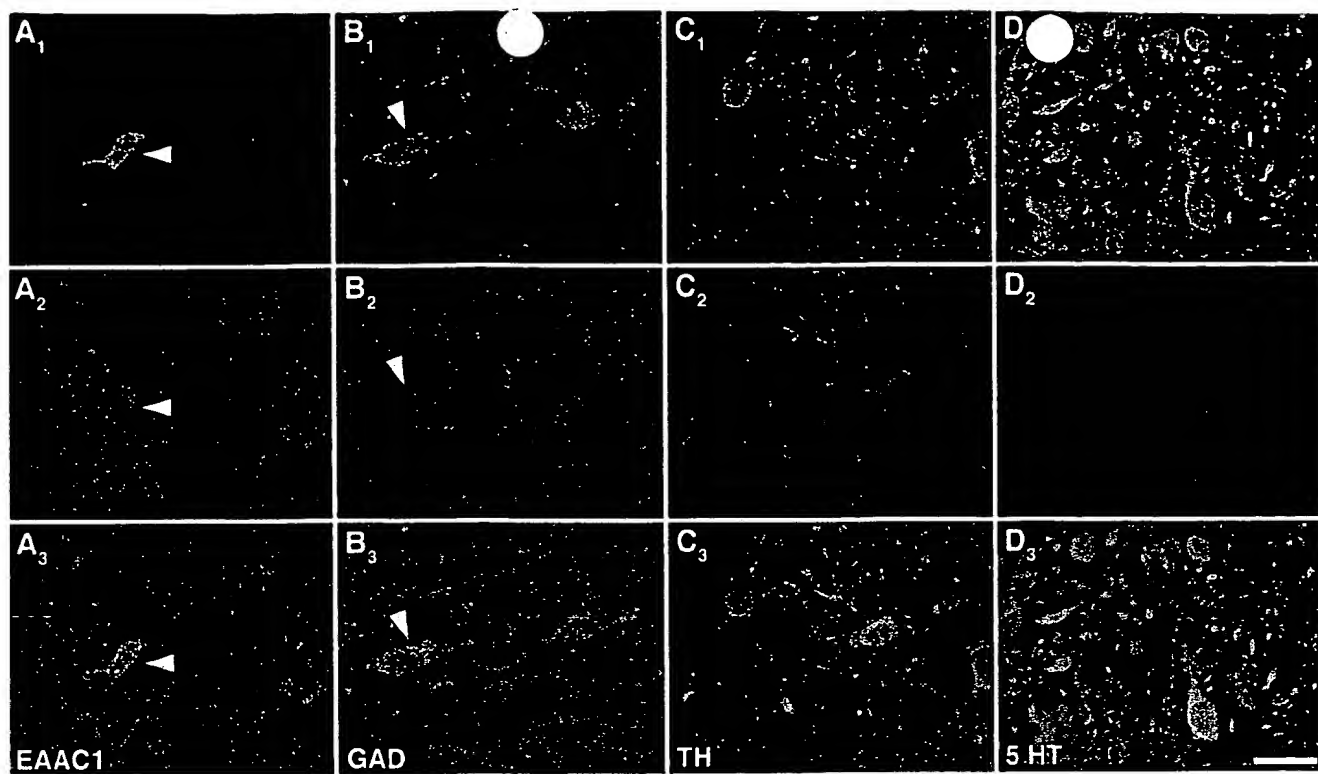


FIGURE 2

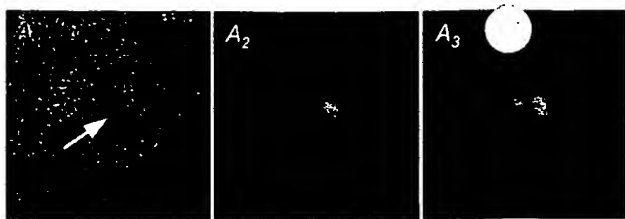
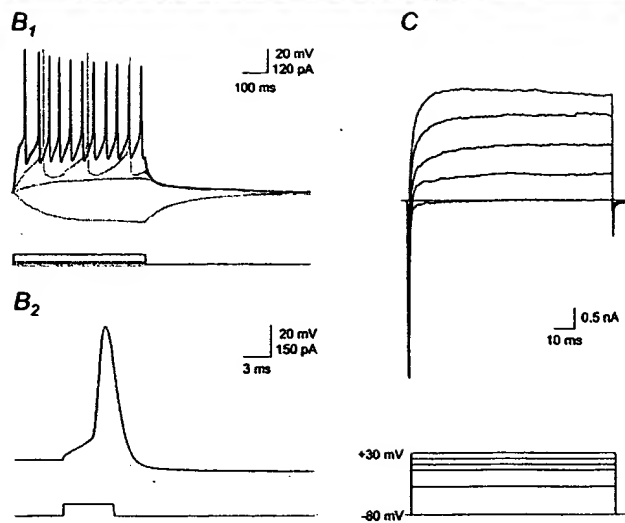


FIGURE 3



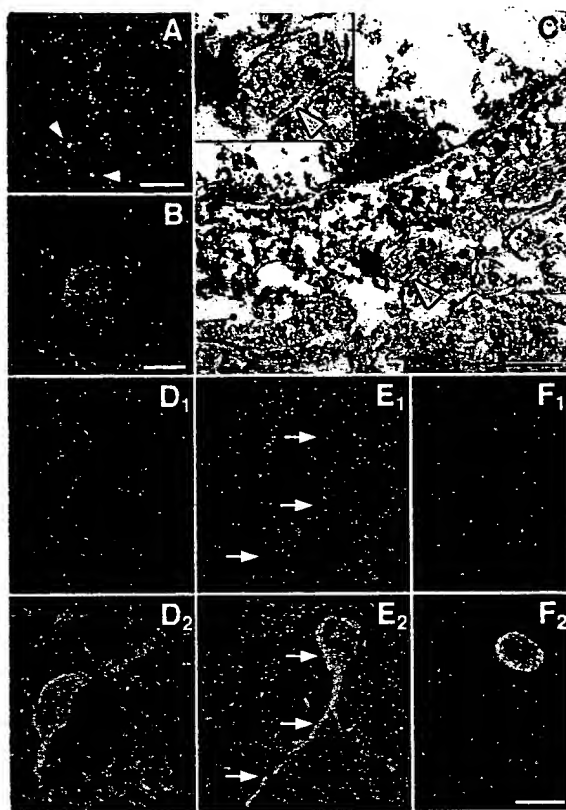


FIGURE 4

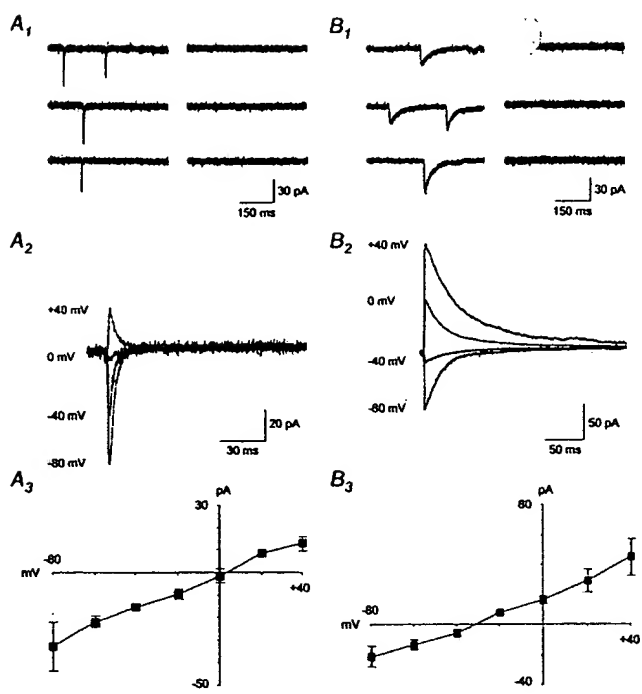


FIGURE 5

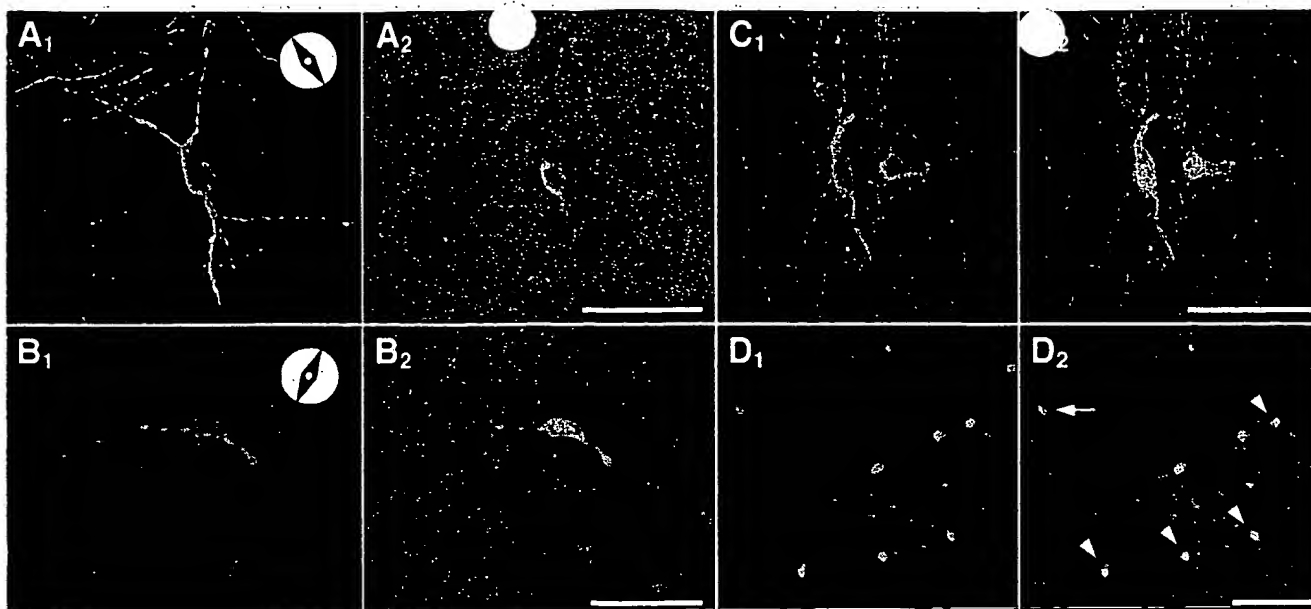


FIGURE 6